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Fumonisins: current research trends in developmental toxicology

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Abstract

Fumonisin B_1 (FB_1) is a mycotoxin produced by *Fusarium verticillioides* that is found in maize and maize-based foods. Reproductive studies in CD1 mice, rats and rabbits initially found no evidence that fumonisins are teratogenic. However, more recent findings suggest that they might increase the risk of neural tube defects (NTDs) in populations consuming large amounts of fumonisin-contaminated corn. When $\geq$15 mg/kg body weight fumonisin B_1 (FB_1) was given to pregnant LM/Bc mice by intraperitoneal (ip) injection, all litters were positive for NTDs. To determine if NTD induction is unique to the inbred LM/Bc mouse strain, NTD induction in LM/Bc and CD1 mice was compared: (a) in a study in which *F. verticillioides* culture material providing $\leq$150 ppm FB_1 was fed to female mice before and during gestation, and (b) in a study in which FB_1 was given by ip injection to CD1 dams on gestation days 7 and 8, the critical time for NTD development. In the feeding study, one of five LM/Bc litters from dams fed the 150 ppm FB_1 diet was positive for NTDs whereas no NTDs were found in the CD1 litters. In the ip injection study, 40% of the litters at the highest dose tested, 45 mg/kg body weight, were positive for NTDs and one of nine low-dose (15 mg/kg body weight) litters was also positive. Thus, FB_1 induced NTDs in both LM/Bc and CD1 mice although the latter strain appears less sensitive. Comparative investigations using these strains will be useful for elucidating the mechanisms underlying fumonisin-induced NTDs in mice and determining the suitability of mouse models for studying the relationships between fumonisins and NTDs in humans.

Keywords: fumonisins, *Fusarium verticillioides*, developmental toxicology, neural tube defects

Introduction

Fumonisins are produced by *Fusarium verticillioides* (formerly *F. moniliforme* Sheldon) and other *Fusarium* species (1, 2). They occur in maize worldwide (2) and in home-made and commercial maize-based foods (3). Fumonisins have also been found in other commodities including cowpeas (4), asparagus (5), sorghum (6), rice (7), and farro (8).

Fumonisin B_1 (FB_1) is a cause of equine leukoencephalomalacia and porcine pulmonary edema, animal diseases resulting from the consumption of *F. verticillioides*-contaminated feeds (2). FB_1 is hepato- and nephrotoxic to laboratory animals (1, 2, 9) and nephro- and hepatocarcinogenic in rodents (10, 11). Human health effects of *F. verticillioides* and fumonisins are unclear, however, they might pose a risk for cancer and other diseases (2). The Transkei, southern Africa and Linxian Province, China are regions where consumption of *F. verticillioides* contaminated-maize and esophageal cancer rates have been correlated (reviewed by Bolger *et al.* (2)). Rising consumption of corn-based products contaminated with fumonisins and increasing esophageal cancer incidences in Italy (12, 13) have been reported and *F. verticillioides* is prevalent in maize from a region in Iran having high esophageal cancer rates (14). FB_1 is categorized "possibly carcinogenic to humans" (Class 2B) by the International Agency for Research on Cancer (15). Fumonisin exposures in Europe and North America are generally low but can exceed the provisional maximum tolerated daily intake (MTDI) of 2 μg/kg body weight in groups consuming large amounts of maize products for health (*e.g.* gluten intolerance) or cultural reasons (2). Guidance levels for fumonisins in maize-based food products in

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Table 1. Recommendations for allowable concentrations of fumonisins in maize and food products in the United States and Europe

United States (43, 44)	
Item	Industry Guidance (ppm)[a]
Whole or partially degermed dry milled maize products[c] (fat content ≥2.25% dry weight)	4
Degermed dry milled maize products[c] (fat content <2.25% dry weight)	2
Dry milled maize bran	4
Cleaned maize for popcorn	3
Cleaned maize for masa	4
Europe (45)	
Item	Maximum Level (ppm)[b]
Unprocessed maize[d]	2
Maize grits, meal, flour and refined semolina	1
Processed maize-based foods for infants and young children and baby food[e]	0.2
Other maize-based products for direct consumption	0.4

[a] Total fumonisins; defined as the sum of fumonisins $B_1 + B_2 + B_3$
[b] The sum of fumonisins $B_1 + B_2$; designated levels go into effect after October 1, 2007 unless other levels are set beforehand
[c] Dry milled maize products include flaking grits, maize grits, meal and flour
[d] Includes maize that is cleaned, sorted and dried before introduction into the market for further processing by thermal or physical methods
[e] Value applies to the dry matter in processed cereal food products and baby food for infants and young children

the United States were established in 2001 and maximum allowable levels for fumonisins in maize and maize based foods have been proposed by the European Union (Table 1).

Initial laboratory animal studies provided no evidence that *F. verticillioides* and FB_1 are teratogenic (Table 2). However, more recent observations (16) and experimental findings (18, 19) have again drawn attention to fumonisins as a possible risk factor for birth defects, specifically neural tube defects (NTDs). The evidence implicating fumonisins is reviewed and preliminary results from ongoing research on the role of fumonisins in NTDs, using the mouse as an animal model, are summarized below.

Fumonisins, sphingolipid metabolism and mechanism of toxicity

Fumonisins inhibit ceramide synthase, thereby blocking *de novo* ceramide synthesis and disrupting sphingolipid metabolism (19, 20). As a result, the ceramide substrates sphinganine and sphingosine accumulate in tissues, serum, and urine and the concentrations of complex sphingolipids derived from ceramide decrease. Increased sphinganine concentration and sphinganine to sphingosine ratio in tissues, serum and urine are utilized as useful biomarkers of exposure in laboratory investigations (Figure 1).

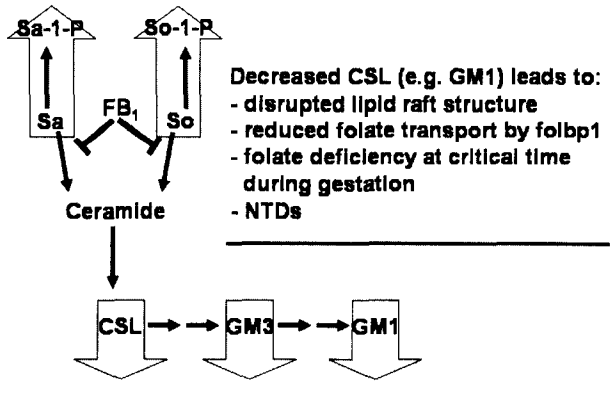

Figure 1. Hypothesis linking disruption of ceramide synthase by fumonisin B_1 (FB_1), to disrupted sphingolipid metabolism and decreased complex sphingolipids (CSL, *e.g.* GM1). Decreased CSL leads to disruption of membrane lipid raft structure, inhibition of the lipid raft associated folate transporter (folbp1), decreased folate utilization and deficiency at a critical time during gestation, and neural tube defects (NTDs) in mice. Increased tissue sphinganine (Sa, intermediate in *de novo* ceramide biosynthetic pathway), sphingosine (So, product of ceramide degradation) and their 1-phosphate metabolites (Sa-1-P, So-1-P) are biomarkers of fumonisin exposure (20). Hypothesis is based on the findings of Gelineau van-Waes *et al.* (17), Sadler *et al.* (27), and Stevens and Tang (31)

Table 2. Summary of selected Fumonisin reproductive and teratology studies

Species	Maternal Dosing	Noteworthy Findings: Maternal	Fetal	Reference
SD Rat	*F. verticillioides* culture material feeding study Dietary FB_1 levels 0, 1, 10 & 55 ppm. Feeding began 2 weeks before mating	Maternal toxicity at 55 ppm (serum chemistry and histopathology) Hepatic sphinganine (Sa)/sphingosine (So) ratio increased on GD15 at 55 ppm	No physical anomalies found (GD15). Tissue Sa/So not increased at any dose level. No distribution of ^{14}C-FB_1 (iv injection to dams on GD15) to fetuses	Voss *et al.* (32)
F344 Rat	0, 30 or 60 mg/kg body weight (BW) FB_1 by gavage given GD8-12	None noted (histopathology, tissue Sa or Sa/So ratio and serum chemistry data not reported)	Decreased litter weight and delayed ossification of vertebrae and sternebrae, 15 and 60 mg/kg. No teratology findings	Lebepe-Mazur *et al.* (33)
F344 Rat	0 and various ranging from 1.9-50 mg/kg BW given on GD3-16	Increased renal Sa/So ratio at $\geq$1.9 mg/kg BW. Decreased kidney weights at doses $\geq$3.8 mg/kg. Kidney lesions at doses $\geq$6.3 mg/kg BW	No teratologic effects; increased number of soft tissue variations found at doses $\geq$3.8 mg/kg BW, but no dose-related increase in one any type of variation. No Sa or Sa/So effects at $\leq$50 mg/kg BW. Fetal effects judged secondary to maternal toxicity	Collins *et al.* (34, 35)
CD1 Mouse	0 and incremental from 12.5 - 100 mg/kg BW by gavage on GD7-15	Maternal toxicity at $\geq$25 mg/kg BW (histopathology, serum chemistry, increased liver Sa/So, decreased body weight or mortality)	Fetal toxicity at $\geq$25 mg/kg (fetal death, decreased fetal weight) secondary to maternal toxicity but no teratological effect. Fetal Sa/So not increased	Reddy *et al.* (36)
New Zealand White Rabbit	0, 0.1, 0.5 and 1.0 mg/kg BW by gavage on GD 3-19	Increased tissue Sa/So; maternal toxicity at $\geq$0.5 mg/kg	Decreased (fetal) body, liver or kidney weight at $\geq$0.5 mg/kg BW. No effect on fetal Sa or Sa/So found. No evidence of significant developmental effects in presence of maternal toxicity	LaBorde *et al.* (37)
Syrian Hamster	0, 12, 18 mg/kg BW by gavage on GD8-9	None reported (tissue Sa and Sa/So not investigated)	Increased fetal death and decreased fetal weight; one litter each with ectodactyly or curled/hooked tail	Floss *et al.* (38)

Accumulation of the sphingoid base 1-phosphate metabolites can also occur and increased concentrations of sphinganine 1-phosphate were recently demonstrated in the kidney of fumonisin-exposed rats (Riley, unpublished observations).

Sphingoid bases and their 1-phosphate metabolites, ceramide and complex sphingolipids are important molecules regulating apoptosis, the cell cycle, and other vital cell functions (19, 20). Sphingosine 1-phosphate is particularly important in extracellular signaling for cell differentiation and mitogenesis and works by activating G-protein-coupled sphingosine 1-phosphate receptors found in cell membranes (21). Sphingosine 1-phosphate is also involved in intracellular signal transduction for calcium mobilization (21). FB_1 has been shown to interfere with cell to cell or cell-matrix adhesion and membrane permeability, likely through mechanisms involving gangliosides (22), sphinganine (23) or sphingoid base 1-phosphates (24). Aside from sphingolipid-mediated events, other signaling pathways and molecular mechanisms might also be involved in fumonisin-induced apoptosis, cell cycle disruption, cell proliferation, and toxicity. Among these are tumor necrosis factor α, NF-κB, fas-ligand, and cyclin D (25, 26) or oxidative injury and changes in fatty acid metabolism and composition (26). The molecular mechanisms underlying fetotoxicity and, in the mouse, NTD induction by FB_1 are not defined and it is likely that, in addition to the aforementioned possibilities, other factors may be critical.

Early experiments on fumonisins and developmental toxicity

Results of earlier studies of fumonisin-producing *Fusarium* and FB_1 indicated that they are fetotoxic but not teratogenic in rodents and rabbits (Table 2). In addition, no direct (biodistribution of radiolabeled FB_1) or indirect (altered sphingoid base biomarkers in fetal tissue) evidence for *in utero* fumonisin exposure was found in these investigations. This is important because FB_1 induced NTDs in mouse embryos (27) and hydrolyzed FB_1 (the alkaline hydrolysis product of FB_1 found in masa and tortillas (3)) caused NTDs in presomite rat embryos (28) *in vitro* (embryo culture). Thus, it was concluded that the placenta provided a barrier preventing *in utero* exposure to fumonisins.

Epidemiological and experimental evidence that fumonisins are a risk for NTDs

Neural tube defects (NTDs) are congenital malformations arising when the embryonic neural tube fails to close properly during gestation (17). NTDs include the phenotypes spina bifida, exencephaly and craniorhachischisis (fission and externalization of the brain and spinal cord) and are, except in the mildest forms, incompatible with life or lead to infection of exposed neural tissue and death of the newborn. NTDs are relatively common: their overall incidence averages about 10/10,000 live births (18, 29).

Among Mexican-American women living in the counties of the Texas-Mexican border region, the NTD rate is about 15/10,000 live births (18), however, it rose to 27/10,000 live births in 1990-91. Hendricks (16) hypothesized that fumonisins, which were found in high concentrations in locally-grown maize at that time, were involved and Missmer *et al.* (unpublished results summarized in Marasas *et al.*, (18)) suggested an association between the increased likelihood of an NTD-affected pregnancy in the affected population and the consumption of homemade tortillas, possibly contaminated by fumonisins. High NTD rates also occur in areas of southern Africa (35-61/10,000), rural northern China (57-73/10,000), and Guatemala (up to 106/10,000 in some areas) where maize is a dietary staple and is likely to be contaminated with fumonisins (2, 18). NTD etiology is poorly understood but is complex and involves genetic, nutritional, environmental, and other factors (16, 29, 30). Folate is particularly important and dietary folic acid supplementation before and during pregnancy reduces the risk of NTDs (30).

FB_1 inhibited carrier-mediated transport of folate in CaCo-2 cells (31) *in vitro*. This is significant because the high-affinity placental folate carriers (folate binding protein 1 or folbp1 in mice and folate receptor α in humans) are GPI-anchored proteins associated with sphingolipid-rich lipid rafts in cell membranes. These results also provide a plausible mechanism by which fumonisins could indirectly cause NTDs, that is, by a mechanism not requiring *in utero* exposure. The hypothesized sequence of events involves ceramide synthase inhibition, decreased complex sphingolipids in lipid rafts, and disrupted foblp1 function leading to fetal folate

deficiency at the critical time for neural tube closure (Figure 1).

Exposing neurulating mouse embryos to ≥2 μM FB_1 *in vitro* for 26 hours disrupted sphingolipid metabolism, inhibited embryo growth and caused NTDs (27). Furthermore, addition of 1 mM folinic acid to the incubation medium improved embryo growth and reduced NTD incidence from 25 to 9 percent (FB_1 concentration =25 μM), from 27 to 8 percent (50 μM FB_1) and from 48 to 14 percent (100 μM FB_1). When the cultured embryos were exposed to higher (50 μM for 2 hours) FB_1 concentrations, 67 percent were NTD positive and 83 percent exhibited neural crest cell-related craniofacial malformations. The addition of folinic acid to the medium significantly reduced the incidences of NTDs and facial defects and improved embryo growth in this second experiment, thereby supporting the mechanistic hypothesis that fumonisins cause NTDs through folate or folate receptor-dependent mechanisms.

In vivo *studies using mouse models: results and questions*

Dose-dependent increases in the number of litters affected and individual fetuses exhibiting NTDs were induced by intraperitoneal injection of FB_1 to pregnant mice of the inbred LM/Bc strain on gestation days 7.5 and 8.5 (17). The percent of litters (n=10 litters per dose level) having NTD-affected embryos rose from 40 at a maternal dose of 5 mg/kg body weight to 100 at ≥15 mg/kg body weight. The number of NTD positive fetuses per group also increased in a dose-dependent manner, reaching 80 percent at the highest maternal dose tested, 20 mg/kg body weight. Sphingoid base measurements and ^{14}C-FB_1 biodistribution studies also provided data leading to the conclusion that, in contrast to earlier reports (see Table 2), *in utero* exposure to FB_1 can occur.

Furthermore, accumulation of radiolabel in the embryos of dams given ^{3}H-folate was markedly decreased by FB_1 (20 mg/kg body weight) treatment and exposure to 50 mg/kg body weight per day folate during gestation significantly reduced NTD incidence in the litters of FB_1-exposed dams. When administered during early gestation (10 mg/kg body weight on gestation days 6.5-9.5), the complex sphingolipid GM1 prevented FB_1-induction of NTDs more effectively than folate: at a maternal FB_1 dose of 20 mg/kg body weight, 50 percent of the fetuses from folate-supplemented dams had NTDs whereas only 5 percent from the GM1-supplemented dams were NTD positive. Immunohistochemical studies further revealed that GM1 and the Folbp1 were co-localized in the yolk sac membrane and embryonic neuroepithelium and were reduced by maternal FB_1 exposure. Together, these results demonstrated that NTDs can be induced by FB_1 *in vivo* under some conditions and provided further indications that complex sphingolipid depletion and reduced folate uptake were mechanistically involved.

It is not known why the outcomes of the LM/Bc mouse studies differed from those of Reddy *et al.* (36), who did not find NTDs or evidence of *in utero* exposure when FB_1 was given orally to pregnant CD1 mice. One possibility is the existence of strain-specific differences in placental function and it is noteworthy that the *in utero* FB_1 exposure experiments in LM/Bc mice were conducted earlier in gestation (gestation day 7.5 and 8.5) than those that gave negative results (after gestation day 15). This raises the possibilities that placental permeability to fumonisins varies by strain or that it changes as the placenta matures. Physiological differences between the two strains affecting maternal toxicity, embryonic development *per se*, or folate uptake and utilization are also possible. Furthermore, it can be speculated that the outcomes were a function of the different dosing protocols. Reddy *et al.* (36) gave FB_1 by gavage and, if gastrointestinal absorption of fumonisin in mice is low, as has been shown in rats (2, 9), then oral dosing on gestation day 7 might not have allowed sufficient time for disruption of some critical function, such as folate receptor function to occur before neural tube closure.

Current research on NTD development in mice

Experiments comparing the developmental toxicity of FB_1 in the LM/Bc and CD1 mouse strains are ongoing. The underlying hypothesis rests on the assumptions that (a) disruption of folate utilization, which is secondary to sphingolipid metabolism disruption, is critical for NTD development (Figure 1) and (b), as suggested above, oral dosing of FB_1 using the protocol of Reddy *et al.* (1996) might not have allowed enough time for reducing complex

sphingolipids to the extent necessary to disrupt folate utilization at the critical window for NTD development.

LM/Bc and CD1 females were fed diets that contained nominal FB_1 concentrations of 50 or 150 ppm, provided by adding *F. verticillioides* culture material to feed (a control group was fed uncontaminated diet). FB_1 intake during the 5 weeks before mating was 1.6-fold higher in the CD1 strain: the low- and high-dose groups had estimated daily intakes of 13 and 39 mg FB_1/kg body weight, respectively, whereas the values for the low- and high-dose LM/Bc groups were 8 and 25 mg FB_1/kg body weight. After the first 5 weeks (FB_1 exposure continued to the end of the study), the females were mated with unexposed males and, after gestation day 16, their litters examined by necropsy. Maternal toxicity was found only at the high dose. Toxicity was confirmed by the presence of microscopic liver lesions that were typical of fumonisin-induced hepatotoxicity in mice (9, 25) and that were of equal severity in the two strains.

One of the five high-dose LM/Bc litters (20%) was NTD positive; one of 10 fetuses in this litter had a NTD. NTDs were absent in the control and low-dose LM/Bc and in all CD1 litters. A trend toward increased late fetal death was found in the CD1 strain. Two of nine high-dose litters (22%) were affected and, within the affected litters, 40-64% of the fetuses were dead. To determine if *in utero* fumonisin exposure had occurred, the concentrations of sphinganine and its 1-phosphate metabolite in fetal livers were measured (LC-mass spectrometry). No significant differences in either were found among groups in the CD1 strain (Figure 2) (39). In contrast, a dose-related increase in fetal liver sphinganine, significant ($p<0.05$) at the high-dose level, was found in the LM/Bc strain and a significant ($p<0.05$) increase in fetal liver sphinganine 1-phosphate concentration was also found in the high-dose LM/Bc fetuses. These preliminary findings suggest that dietary exposure to fumonisins induces NTDs in LM/Bc mice (investigations to confirm are in progress) and that there is indeed a difference in the susceptibility of CD1 and LM/Bc mice to the developmental effects of fumonisins. They also imply: (a) that the no observed effect level for FB_1-induced NTDs by subchronic dietary exposure is $\geq$50 ppm, (b) that fetotoxicity and NTDs occur in the presence of maternal toxicity, and (c)

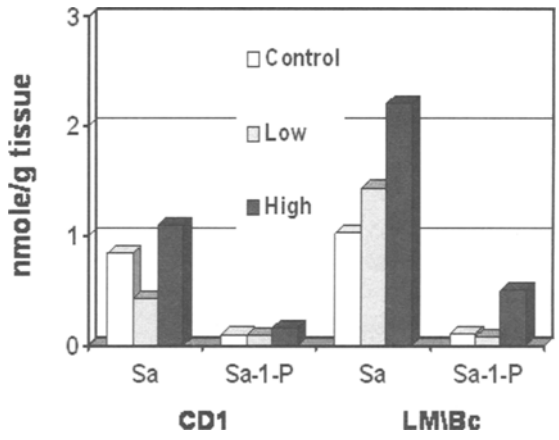

Figure 2. Sphinganine (Sa) and Sa-1-phosphate (Sa-1-P) concentrations in fetal livers from LM/Bc (n=4-5) and CD1 (n=10) litters. The dams were fed diets containing *F. verticillioides* culture material to provide 50 (low dose) or 150 (high dose) ppm fumonisin B_1 beginning 5 weeks before mating and continuing through gestation. Dose-related increases in Sa and Sa-1-P were found in the LM/Bc fetal livers only. Sa and Sa-1-P of the high-dose LM/Bc group were significantly different from their control values, $p< 0.05$. From the data of Riley *et al.* (39)

independently corroborate that *in utero* fumonisin exposure occurs in the LM/Bc strain.

In another experiment, CD1 dams (n=8-10/group) were given intraperitoneal injections of 0, 15, 30 or 45 mg/kg FB_1 dissolved in physiological saline using the dosing protocol of Gelineau-van Waes *et al.* (17). NTDs were found in 4 of 10 high-dose litters and in 1 of 9 low-dose litters. None were found in the control or mid-dose groups. The percent of affected fetuses in the four NTD-positive high dose litters ranged from 3.3 to 33.3 (mean =17). There were also other indications of fetal toxicity. The mean number of viable fetuses per litter decreased at the high-dose level: values were 11.2 (control), 12.3 (low-dose), 13.6 (mid-dose) and 8.7 (high-dose). The mean number of resorptions (includes early and late deaths) per litter concomitantly increased: mean values were 0.8 (control), 1.6 (low-dose), 1.3 (mid-dose), and 2.4 (high-dose). The average mean fetus weight (live fetuses only) of the high-dose group (608 mg) was decreased about 15% compared to the controls (716 mg). Thus, the susceptibility to fumonisin-induced NTDs in mice treated according to the Gelineau-van Waes *et al.* (17) protocol is not unique to the LM/Bc strain, although this strain is significantly more sensitive than the CD1 strain (Figure 3).

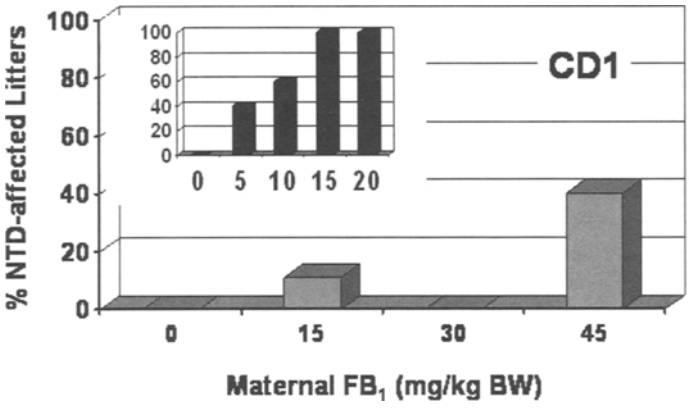

Figure 3. Fumonisin B_1 caused a dose-related increase in the number of litters having one or more fetuses exhibiting exencephaly, a form of neural tube defect (NTD), when given to CD1 and LM/Bc (insert) dams by intraperitoneal injection according to the protocol of Gelineau-van Waes *et al.* (17) (see text for details). CD1 mice were less sensitive in regard to both the number of litters affected and the number of fetuses per group displaying NTDs (see text). LM/Bc data previously reported by Gelineau-van Waes *et al.* (17)

Conclusion

Emerging evidence suggests that fumonisins might be a risk factor for NTDs in populations heavily dependent on foods prepared from fumonisin-contaminated corn, especially if their diets are also deficient in folate. However, the situation is complex as, in addition to fumonisins, other NTD risk factors have been identified in the high-risk Mexican-American population living in the Texas-Mexico border region. These include fever, febrile illness and other sources of hyperthermia (40), periconceptual diarrhea (41), and maternal stress related to life events and social considerations (42). Prospective epidemiological investigations in conjunction with laboratory experiments are needed to further investigate the situation in the Texas-Mexico border region and other areas where NTD incidences and fumonisin exposure are concurrently high, to fully characterize the developmental toxicity of fumonisins, and to develop effective intervention strategies. LM/Bc mice are an established folate and GM1-responsive model for studying the mechanism(s) underlying fumonisin-induced NTDs however, ongoing investigations in CD1 mice have shown that NTD induction in mice is not unique to the LM/Bc strain. Comparative investigations of FB_1-induced developmental toxicity in these two strains should provide a useful tool to elucidate the mechanism(s) of NTD induction in mice and determine their relevance to humans.

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